Generation of recombinant influenza virus using Baculovirus delivery vector

TECHNICAL FIELD

The present invention is in the fields of recombinant gene technology and vaccine development and relates to a method for rescuing recombinant viruses, particularly recombinant orthomyxoviruses, using baculovirus as a gene shuttle for delivery of desired viral genes to a mammalian host cell for expression. It further relates to a modified baculovirus genome as used in said method, and further to the manufacture of vaccines using said method for rescuing recombinant viruses.

BACKGROUND OF THE INVENTION

One of the most important and best characterized members of the orthomyxoviruses is influenza virus. The genome of influenza virus is single stranded RNA of negative polarity that comprises approximately 13500 nucleotides. The genome of both influenza A and B viruses is distributed into eight different segments, coding for nine structural proteins and two non-structural proteins (NS1, NS2) with regulatory functions. The first 12 nucleotides at the 3' end and the first 13 nucleotides at the 5' end of each genome segment of influenza A viruses are conserved in all eight segments. Due to the segmented nature of the viral genome genetic reassortment takes place when mixed infection of two different influenza strains occurs (i.e. avian influenza A strains can reassort with human influenza A viruses).

Several reverse-genetic systems have been developed to allow manipulation of the viral genome. One system is based on the principle that synthetic influenza vRNA was transcribed in vitro and encapsulated with the ribonucleoprotein complex (RNP), consisting of the three viral polymerase proteins (PB1, PB2, PA) and the nuclear protein (NP). Infection with influenza helper virus of RNP transfected cells provides the remaining viral proteins and RNA segments. Using a strong selection system it was possible to isolate virus progeny carrying the recombinant gene (1, 2).

Reverse Genetics

Reverse genetics for negative-strand viruses, first developed for influenza A virus by Luytjes et al (3), has significantly contributed to our biological understanding of these

pathogens and still gives rise to improving the methods of vaccine development. The manipulation of the influenza genome by reverse genetics has led to the possibility of preparing stable laboratory strains which contain site-directed mutations that can be designed in a way such that they confer attenuation in one or more genes. For example, establishment of NS gene engineering methods for influenza A virus allowed to discover the main function of the NS1 protein as interferon antagonist and permitted obtaining genetically stable attenuated strains thatcan be used as a live influenza vaccine (4, 5).

Plasmid-Based Technology

Recently a new plasmid-based technology has been developed for rescuing recombinant influenza A viruses. Co-transfection of 8 to 18 plasmids in a mixture expressing all influenza genomic fragments and proteins required for their transcription allowed to obtain infectious influenza A viruses in the absence of any natural helper strains (6, 7). The advantage of this strategy is that selection for reassortants is no longer required, since only the genes of interest are being expressed. However, one obvious disadvantage of this system is the necessity to use many plasmids for transfection and the low probability of their simultaneous appearance in a transfected cell. As the genetically modified genes can only be introduced by plasmid delivery, transfection efficiency plays a major role in a successful rescue of recombinant influenza virus progeny. In addition, according to our knowledge, only few cell lines can effectively be used in such transfection protocols.

Baculovirus as Gene Delivery Tool

Current methods of expressing genes in a mammalian cell include the use of viral vectors, such as those which are derived from retroviruses, adenoviruses, herpes viruses, vaccinia viruses, polio viruses, sindbis viruses, or adeno-associated viruses. Other methods of expressing an exogenous gene in a mammalian cell include direct injection of DNA, the use of ligand-DNA conjugates, the use of adenovirus-ligand-DNA conjugates, calcium phosphate precipitation, and methods which utilize a liposome- or polycation-DNA complex. Also, viruses of the family Baculoviridae (commonly referred to as baculoviruses) have been used to express exogenous genes in insect cells and mammalian cells. One of the most studied baculoviruses is the *Autographa californica multiple nuclear polyhedrosis virus* (AcMNPV).

The transduction of mammalian cells by baculovirus particles bears no safety risk for either product or operator. The expression of the exogenous genes is transient, unless a G418-resistance-gene is included and selected for by the addition of G418. The transient expression of a mammalian expression cassette present on the baculovirus genome is gene dosage-dependent, corresponding to the number of virus particles used in the experiment. While the transport efficiency of naked plasmid DNA to the nucleus is dependent on the sequence and structural features of the plasmid and therefore less reproducible within different gene constructs, the entire baculovirus capsid containing the target genes gets efficiently and essentially without alterations tranferred into the nucleus, independently of the genetic structure of the contained gene constructs.

BRIEF DESCRIPTION OF THE INVENTION

The invention relates to the construction of one or more baculovirus clones that are able to express one or more, optionally all, genes necessary for the rescue of infectious influenza virus. It is preferred that the baculovirus is a nuclear polyhedrosis virus and most preferably is AcMNPV. Baculoviruses possess several advantages that make them an attractive method for influenza genetic manipulation. Multiple genes and large inserts (up to 38 kb) can be introduced into the baculovirus genome so that one or more or even all influenza genes can be delivered to Vero cells at once. The baculoviruses are inherently unable to replicate in mammalian cells and are therefore considered biologically safe for therapeutic applications for humans.

According to the present invention, a more simple, highly efficient alternative method using baculovirus as a delivery vehicle was developed. The baculovirus *Autographa californica nuclear polyhedrosis virus* (AcNPV) is an insect virus with a large double-stranded circular DNA genome. Although baculoviruses have primarily been used to over-express proteins from insect derived host cells, they have been demonstrated to serve as a powerful vector to carry various genes into a variety of mammalian cell types at high frequencies (8). We investigated the use of baculovirus as an influenza gene delivery vector. Recombinant baculovirus harboring a truncated influenza A NS gene, resulting in a 38 amino acid long NS1 protein, was constructed. This truncation of NS1 mediates an irreversible attenuation of influenza A virus (4) and is considered to be a valuable tool for safe influenza vaccine development. Further, green

fluorescent protein (GFP) was used as a reporter gene and included into the baculovirus genome. The efficiency of baculovirus-based transduction of Vero cells with influenza genes was monitored by GFP expression.

In one embodiment, subsequent infection of baculovirus-transduced Vero cells with a temperature-sensitive (*ts*) influenza helper virus, whose NS gene segment was found to be responsible for the *ts* phenotype, and subsequent passages in Vero cells at 40 °C resulted in the isolation of an influenza virus carrying the baculovirus-derived truncated NS1 gene. This virus, designated ΔNS38, did not grow on Madin-Darby canine kidney (MDCK) cells or in eggs, but gave almost equal titers, compared to the wild type variant, in interferon deficient Vero cells (4).

The invention further relates to a baculovirus genome that contains one or more uni- or bidirectional expression cassettes encoding at least one natural or modified gene or gene segment or a combination of a least one natural and at least one modified gene or gene segment, of an orthomyxovirus. In a preferred embodiment the invention relates to Baculovirus genome wherein said at least one natural or modified gene or gene segment is from influenza A or B virus.

In yet another emodiment, the invention relates to a baculovirus genome wherein said at least one natural or modified gene or gene segment encodes viral mRNA or viral genomic RNA, or both viral mRNA and viral genomic RNA.

In yet another emodiment, the invention relates to a baculovirus genome wherein said at least one natural or modified gene or gene segment is flanked by restriction sites that allow for introduction and exchange of said gene or gene segment by cleavage of said restriction sites and subsequent direct ligation of said gene(s) or gene segment(s) into the baculovirus genome. These restriction sites are unique in baculovirus.

In yet another emodiment, the invention relates to a baculovirus genome wherein said at least one natural or modified gene or gene segment comprises a modified NS gene segment of influenza virus encoding an NS1 protein of only 38 amino acids in length (ΔNS38), wherein the 38 amino acids are the N-terminal starting sequence of the NS1 protein. The big C-terminal truncation of the NS1 amino acid sequence renders the influenza virus interferon inducing and, simultaneously, interferon sensitive, which has in effect that the resulting virus is attenuated (at least due to its susceptibility to IFN activity) and thus a promising candidate for making live vaccines.

In yet another emodiment, the invention relates to a baculovirus genome wherein said at least one natural or modified gene or gene segment comprises a modified NS gene segment of influenza virus, a hemagglutinin (HA) and/or a neuraminidase (NA) gene segment of an epidemic influenza strain, and all remaining gene segments of the epidemic strain or of one or more other influenza wildtype or laboratory master strains.

DETAILED DESCRIPTION OF THE INVENTION

The AcMNPV genome comprises 134 kbp of double-stranded DNA. The capacity of foreign gene insertion into the baculovirus genome has been demonstrated to be at least 40 kbp of DNA. Therefore, it becomes possible to stably integrate more than one gene-expression cassette into the AcNPV genome, e.g. target gene plus reporter gene, protein complexes consisting of multiple subunits.

The baculovirus genome can carry an exogenous promoter positioned for expression of the exogenous gene. Preferred promoters include the cytomegalovirus early promoter (CMV) and the human ribosomal RNA polymerase I promoter (Pol-I). The baculovirus genome may also carry a polyadenylation signal, the bovine growth hormone poly adenylation signal (BGH-Poly A) or the hepatitis delta virus genomic ribozyme (HDV), a self-cleaving RNA sequence which ensures the correct 3' end.

According to the present invention, the baculovirus is engineered to contain as a heterologous insertion into its genome at least one, preferably several, optionally all genes or gene segments of influenza virus, or of any other orthomyxovirus. Influenza A as well as B viruses possess a segmented single-stranded, negative-strand RNA genome. For Orthomyxovirus replication and infection, all RNA-segments must be produced as negative strand viral genomic RNA (vRNA) and four of these RNA segments must be present either as functional proteins or as transcripts encoding proteins that have to be functional for transcription and replication of all segments. According to the present invention, the different Orthomyxovirus genes may be engineered in any combination or number into a single baculovirus genome or may be divided into two or more portions and distributed to two or more separate baculovirus genomes. A baculovirus containing just one foreign gene, e.g. a modified version of the NS1 gene, may be used for transduction of Vero cells and rescue of infectious virus, e.g. influenza virus or any orthomyxovirus, may be carried out via

superinfection with helper virus. Alternatively and as a preferred embodiment, co-transduction of several baculoviruses, each containg one or more different orthomyxovirus genes, may be carried out in order to rescue infectious orthomyxovirus particles, preferably without the aid of helper virus.

The term "transduction" as used herein refers to carrier-mediated transfer of genetic material to a living host cell, wherein the carrier is an enveloped virus and the genetic material is contained within the capsid of said virus, and wherein the transfer is accomplished in a way such that upon contact of the virus with the host cell the capsid containing the genetic material penetrates the host cell and migrates to the host cell's nucleus, while the viral envelope remains outside the host cell. The baculovirus used according to the present invention is such a viral carrier.

The term "transfection" as used herein refers to the transfer, usually carrier-mediated transfer, of naked RNA or DNA, e.g. plasmid RNA or DNA vector constructs, to host cells. Suitable carriers are, for instance, RNP (ribonucleoprotein complex) or lipids (e.g. Lipofectin®).

The term "infection" as used herein refers to the exposure of host cells to natural or modified viruses and the subsequent delivery of viral RNA or DNA to the host cell using natural pathways, including, as the case may be, penetration of the host cell by the whole virus. For example, the term applies to the delivery of influenza virus RNA gene segments to a host cell, e.g. a Vero cell, using an influenza wildtype or helper virus.

Transduction efficiency of Vero cells by Baculovirus.

The efficiency of recombinant baculovirus for gene transfer into Vero cells was investigated. Recombinant AcCMVGFP-ΔNS38 baculoviruses carrying GFP reporter gene at different MOIs in the presence of DEAE dextran sulfate were transduced into the Vero cells. After 48 hours, GFP expressing cells were analysed by FACS analysis. Transduction of Vero cells by the recombinant AcCMVGFP-ΔNS38 baculovirus was concentration-dependent as shown in FIG.2A. MOIs tested were 5, 50, 500 and 5000, yielding transfection rates of 2%, 9%, 62% and 91% respectively. No changes in cell viability and no cytopathic effects of transduced Vero cells were observed.

Rescue of recombinant ΔNS38 influenza virus.

A baculovirus-delivered, truncated NS1 gene from influenza A virus (ΔNS38-gene) was expressed in Vero cells and transcribed vRNA was successfully incorporated into influenza virus particles. To analyze the viral progeny after selection, RT-PCR with PR8 and helper virus-specific primers was performed after plaque purification (Fig. 2). A mixture of the truncated ΔNS38-vRNA and helper virus NS-vRNA was detected after two selective passages at 40°C. After several plaque purification steps, a homogenous virus population could be isolated and the ΔNS38 gene was confirmed by nucleotide sequence analysis.

Growth of ΔNS38 virus in tissue culture.

Recombinant ΔNS38 virus showed similar growth patterns in interferon-deficient Vero cells as compared to the 25A-1 helper virus (a reassortant virus containing the NS segment form the cold-adapted influenza strain A/Leningrad/134/47/57 and the remaining genes from influenza A/PR/8/34) but was fully restricted from growth on MDCK cells or in eggs.

The baculovirus was shown for its effective delivery of foreign genes into Vero cells. Transduction efficiency of the recombinant baculovirus at different MOIs into Vero cells was evaluated, using GFP as a reporter gene. FACS analysis revealed that the Vero cell transduction was concentration-dependent (FIG.2B). More than 90% of the cells were transduced when recombinant baculovirus was used at MOI 5000, as evidenced by their expression of GFP 24 to 48 hours after transduction. Although high numbers of baculovirus particles may be required, this method is considered feasible and significantly superior to the afore-mentioned methods known in the art, since baculoviruses can be easily generated and grown to high titers in insect cells.

We were able to demonstrate the efficient delivery of recombinant, truncated NS genes into influenza virus using baculovirus as a delivery vehicle. The truncated NS1 protein of this virus, consisting of only 38 N-terminal amino acids, is responsible for the attenuated phenotype in mice, which is based on the finding that ΔNS38 virus has an interferon inducing but also an interferon sensitive phenotype.

Using the method of the present invention for the rescue of attenuated epidemic viruses, particularly influenza viruses, appears to be a promising way to obtain candidates for a live influenza vaccine and for the rapid and efficient manufacture of attenuated live vaccines.

In a preferred embodiment of the invention, some or all genes of the desired recombinant Orthomyxovirus, e.g. of a desired attenuated and/or recombinant influenza virus, are inserted into the genome of a single baculovirus and thereafter transduced into Vero cells for expression of the viral genes and for assembly and release of the desired recombinant Orthomyxoviruses. Alternatively and equally preferred from a technical point of view, some or all of the genes or gene segments, respectively, of the desired recombinant virus are divided into two portions and distributed into the genomes of two baculoviruses and thereafter co-transduced into Vero cells for expression and assembly of recombinant virus.

The term "desired" virus shall mean any virus having a pre-selected phenotype, preferably having an attenuated (e.g. temperature-sensitive, interferon-sensitive, or otherwise replication-inhibited) phenotype, and/or a pre-selected set of genes or gene segments. For instance, it is preferred that the desired virus be an influenza virus that comprises a modified NS gene segment responsible for interferon induction and/or for interferon sensitivity such as the ΔNS38 gene segment (coding for a full NEP and a truncated NS1 protein of only 38 amino acids length), a hemagglutinin (HA) and/or a neuraminidase (NA) gene segment of an epidemic influenza virus strain, and some or all of the remaining gene segments of one or more other influenza wildtype or laboratory master strains.

The present invention also encompasses an embodiment, wherein a part or the entirety of genes or gene segments, respectively, of a desired recombinant virus, are split into more than two portions and distributed to more than two baculovirus genomes, each baculovirus genome receiving just one of said more than two portions of genes or gene segments, respectively. The recombinant baculoviruses produced by this method and differing from one another by the inserted portion of foreign genes or gene segments are then co-transduced to the host cells, preferably Vero cells, for expression of the viral genes and assembly and release of the desired recombinant

virus particles. Both methods have in common that they do not require the use of a helper virus to rescue the desired recombinant virus.

Where it is preferred to transduce just some of the viral genes of a desired recombinant virus into the host cell expression system, e.g. into Vero cells, it will be required to supply the remaining viral genes by other methods, for instance by infecting the transduced host cells with a helper virus. In doing so, some of the newly generated virions will be modified helper viruses, i.e. helper viruses wherein the transduced genes or gene segments replace the corresponding genes or gene segments of the helper virus. Although this method requires the employment of a helper virus and a suitable subsequent selection system for rescuing the desired recombinant reassorted viruses (i.e. the modified helper viruses), it is still more efficient than all other reverse genetic methods known to us so far.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Construction of recombinant NS expressing cassette.

The NS gene was cloned in reverse orientation between human polymerase I promoter (PolI) and hepatitis delta virus terminator (HDV) to transcribed NS vRNA. At nucleotide position 140, a multiple stop codon cassette was introduced. resulting in a truncated, 38 amino acid NS1 protein and a full size, 121 aa NEP. The non-translated part between the stop codon and the splicing signal of NEP was deleted.

Fig. 2: Expression of the reporter gene (GFP) in infected Vero cells depending upon baculovirus concentration.

A) Vero cells were transduced with baculovirus carrying a GFP reporter gene at different MOIs. Using FACS analysis, the percentage of GFP expressing cells was measured (i.e. reflecting cells are transduced with baculovirus).

B) Vero cells expressing GFP after 48 hours transduction with recombinant AcCMVGFP-ΔNS38 baculovirus.

Fig. 3: Detection of the recombinant influenza NS gene.

Virus-containing supernatant, obtained from Vero cell culture after transduction with baculovirus and subsequent infection with influenza helper virus, was passaged twice at 40°C and analyzed by RT-PCR, using two sets of primers which selectively

detect only wild-type (lane 1) or only recombinant, truncated gene (lane 2) In picture A, the supernatant comprises a mixture of helper virus 25A-1 and rescued ΔNS38 virus (Fig. 3A). After several plaque purification steps a pure recombinant ΔNS38 virus was isolated (Fig. 3B).

Example 1.

Cell culture.

Spodoptera frugiperda (Sf9) insect cells were grown in IPL-41 insect medium (Sigma-Aldrich Chemical) containing yeastolate and a lipid/sterol cocktail with 3% FCS at 27°C. Vero (WHO-certified) cells were grown in DMEM/Ham's F12 (Biochrom) protein free medium.

Influenza virus.

Influenza virus 25A-1 is a temperature-sensitive *(ts)* reassortant containing the NS gene from the cold-adapted strain A/Leningrad/134/47/57 (H2N2) and the remaining genes from the A/Puerto Rico/8/34 (H1N1) virus. The NS gene is responsible for the temperature-sensitive phenotype of the helper virus. The virus was amplified in Vero cells in serum free medium.

Construction of recombinant AcCMVGFP-ΔNS38 baculovirus.

The NS gene was cloned between human ribosomal polymerase I promoter (PolI) and hepatitis delta virus genomic ribozyme (HDV), a self-cleaving RNA sequence which ensures the correct 3' end. A multiple stop codon cassette was introduced after the nuclear localization signal 1 (NLS1) of the NS1 protein. The non-translated part between this stop codon cassette and the splicing signal of the nuclear export protein (NEP) was deleted and resulted in a truncated NS1 protein, consisting of only 38 amino acids but a fully intact NEP protein (6). The NS construct, designated ΔNS38 was inserted into a baculovirus transfer vector (pCMVGFP), a modified pBacPAK8 (Clonetech), expressing the green fluorescence protein (GFP) under control of the cytomegalovirus promoter (CMV) and the bovine growth hormone polyadenylation site (BGH) as reporter-gene. The recombinant plasmid (pCMVGFP-ΔNS38) and linear *Ac*NPV viral DNA (Baculogold, PharMingen) were co-transfected into *Spodoptera frugiperda* (*Sf9*) insect cells and recombinant AcCMVGFP-ΔNS38

baculoviruses were generated. The recombinant AcCMVGFP-ΔNS38 baculoviruses were purified by ultracentrifugation and resuspended in serum-free Vero cell medium.

Generation of recombinant ΔNS38 influenza viruses.

Recombinant baculovirus inoculum (MOI=1000) with DEAE-dextran (1:100) was added to Vero cells and incubated for two hours, followed by replacement of the inoculum with serum-free medium. After 24 hours, the baculovirus-transduced cells were infected with 25A-1 influenza helper viruses at MOI of 1 for 30 minutes. The helper virus inoculum was replaced with serum-free medium and the Vero cells were further incubated at 37°C for 24 h. The viral supernatant was passaged twice in Vero cells at 40°C and analyzed by RT-PCR followed by nucleotide sequence analysis for the presence of the ΔNS38. Following three rounds of plaque purification on Vero cells, recombinant influenza viruses containing the ΔNS38-vRNA were isolated (Fig. 3).

Transduction efficiency of baculovirus in Vero cells.

Vero cells were transduced with recombinant AcCMVGFP-ΔNS38 baculovirus, carrying a GFP reporter gene at different MOIs using the procedure described above. After 48 hours, the transduced cells were trypsinized and resuspended in PBS. GFP expressing cells were analyzed by a fluorescence activated cell sorter (FACS).

Example 2.

Simultaneous co-transduction of four different gene segments.

Influenza B virus genes of strain B Lee 40 were released from the cloning vector backbone pHW2000 by restriction digests and inserted into the baculovirus transfer vector pBacPAK8. More particularly, the three polymerase genes and the gene encoding the nuclear protein of influenza B virus (B Lee 40), were taken from the constructs pHW-Lee-PB1, pHW-Lee-PB2, pHW-Lee-PA, pHW-Lee-NP, which are based on pHW2000 (Hoffmann et al., 2000), kindly received from Thorsten Wolff (Robert Koch-Institute, Berlin) and were inserted each into a baculovirus transfer vector pBacPAK8 (Clontech). In these expression vectors, the genes are expressed bidirectionally, transcription (i.e. generation) of the messenger RNA is driven by the cytomegalovirus (CMV) early promoter and terminated by the bovine growth

hormone (BGH) polyadenylation signal. Transcription (i.e. generation) of genomic RNA is driven by the human polymerase I (Pol I) promoter and terminated by the hepatitis delta virus (HDV) genomic ribozyme, a self-cleaving RNA sequence to ensure the correct 3' end (Neuman et al., 1994). This set of proteins is required for reverse transcription of influenza virus genes and genes that are flanked by influenza virus non-coding sequences, where the nuclear complex binds and initiates transcription.

Recombinant baculovirus clones were generated via homologous recombination using the above described transfer vectors, and amplified and purified by sucrose gradient centrifugation. In order to prove funcionality of the cloned genes, a plasmid was constructed, which contained the green fluorescent protein (gfp) in minus sense orientation, flanked by influenza B virus non-coding sequences. The gene encoding minus sense RNA of green fluorescent protein is expressed under control of the human polymerase I promoter (PolI) and terminated by the hepatitis delta virus genomic ribozyme (HDV).

The RNP-complex comprising the four influenza B virus proteins (PA, PB1, PB2, NP) binds to the non-coding region and initiates transcription. Only when all four influenza virus proteins comprised in the nuclear complex (PB1, PB2, NP, PA) are functionally active hence successfully delivered and expressed, gfp mRNA is being produced and gfp expression can be detected by a fluorescent signal. For this purpose, HEK293 Freestyle cells (Invitrogen) were transduced with a pool of the four baculovirus clones, using a total moi of either 50, 500, 1000, 5000 or 7500. 48 hours post transduction, cells were transfected with 1µg of purified plasmid containing the minus sense gfp gene flanked by influenza B virus non-coding sequences. Analysis was performed 24 hours post transfection by fluorescence activated cell sorting.

It could be shown that gfp was functionally expressed in the HEK293 freestyle cells due to transcriptional activity of the influenza B virus nuclear complex which formed and assembled within the cells upon delivery and expression of the four different baculovirus clones. Thus, the principle underlying the present invention as claimed herein was fully confirmed. Particularly, it was proven that the present invention allows not only for successful delivery of several different genes of interest to a

mammalian host cell using the baculovirus-based transduction system but also for successful expression of the delivered genes.

Analogously, co-transfection of a pool of eight different baculovirus clones each clone comprising another influenza virus gene or gene segment can likewise result in successful transcription and expression of the delivered genes and subsequent assembly of the expressed proteins to form virus particles.

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